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REVIEW

The obscure events contributing to the evolution of an incipient sex chromosome in *Populus*: a retrospective working hypothesis

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Abstract Genetic determination of gender is a fundamental developmental and evolutionary process in plants. Although it appears that dioecy in *Populus* is genetically controlled, the precise gender-determining systems remain unclear. The recently released second draft assembly and annotated gene set of the *Populus* genome provided an opportunity to revisit this topic. We hypothesized that over evolutionary time, selective pressure has reformed the genome structure and gene composition in the peritelomeric region of the chromosome XIX, which has resulted in a distinctive genome structure and cluster of genes contributing to gender determination in *Populus trichocarpa*. Multiple lines of evidence support this working hypothesis. First, the peritelomeric region of the chromosome XIX contains significantly fewer single nucleotide polymorphisms than the rest of *Populus* genome and has a distinct evolutionary history. Second, the

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peritelomeric end of chromosome XIX contains the largest cluster of the nucleotide-binding site–leucine-rich repeat (NBS–LRR) class of disease resistance genes in the entire *Populus* genome. Third, there is a high occurrence of small microRNAs on chromosome XIX, which is coincident to the region containing the putative gender-determining locus and the major cluster of NBS–LRR genes. Further, by analyzing the metabolomic profiles of floral bud in male and female *Populus* trees using a gas chromatography-mass spectrometry, we found that there are gender-specific accumulations of phenolic glycosides. Taken together, these findings led to the hypothesis that resistance to and regulation of a floral pathogen and gender determination coevolved, and that these events triggered the emergence of a nascent sex chromosome. Further studies of chromosome XIX will provide new insights into the genetic control of gender determination in *Populus*.

Keywords Gender determination · Sex chromosome · Single nucleotide polymorphisms (SNP) · MicroRNA (miRNA) · Nucleotide-binding site–leucine-rich repeat (NBS–LRR) · *Populus*

Introduction and background

Genome structure and synteny map across species

Genus *Populus* contains approximately 30 species that occur throughout the northern hemisphere and consists of six subgenera or sections: *Abaso*, *Leuce* (aka *Populus*), *Leucoides*, *Aigeiros*, *Turanga*, and *Tacamahaca* (Eckenwalder 1996). The first investigation of the *Populus* genome was made in 1921, in which the haploid chromosome number was erroneously reported as four (Graf 1921). By 1924, it became clear that the base chromosome number in *Populus* was 19 (Harrison 1924). Since then, examination by various scientists has revealed that all *Populus* species generally appear as diploids with $2n=38$ (Smith 1943), with occasional cases of triploid or tetraploid genets arising naturally in various species though more often reported in members of the *Leuce* subgenera and in interspecific crosses (Einspahr et al. 1963; Bradshaw et al. 2000). Analysis of the assembled genome revealed that the chromosomal structure in modern *Populus* arose from an ancient whole-genome duplication event known as "salicoid" duplication (Fig. 1) in a progenitor that possessed 12 ancestral chromosomes (Salse et al. 2009). Genome organization and chromosome structure have been conserved among *Populus* and *Salix* species (Berlin et al. 2010), and comparisons among *Populus* and *Salix* orthologous genes suggest that both genera share this whole-genome duplication event that predated the speciation event (Tuskan et al. 2006). Comparative mapping reveals near-complete marker colinearity in pedigrees established from multiple species within *Populus* and among members of *Salix* (Cervera et al. 2001; Hanley et al. 2006; Yin et al. 2004a, 2008; Berlin et al. 2010) (Table 1). Interestingly, Berlin et al. (2010) identified a large region of segregation distortion on linkage group XIX that corresponds to a similar region in chromosome XIX in *Populus* (Yin et al. 2008).

Members of the genus *Populus* generally display separate genders on individual trees, i.e., *Populus* which is dioecious (Slavov et al. 2010; Hughes et al. 2000; McLetchie et al. 1994; Nagaraj 1952), as is *Salix* (Karp et al. 2011). Only about 4 % of higher plants are dioecious (Ainsworth 2000; Ming et al. 2007; Heslop-Harrison and Schwarzacher 2011). This reproductive habit in *Populus*, along with the ubiquitous vegetative reproduction via root suckering, air layering, and/or cladoptosis, evolved proximally to or simultaneously with the advent of this family 65 million years ago, as nearly all members of the Salicaceae family displays these habits (Karrenberg et al. 2002; Eckenwalder 1996). Dioecy in *Populus* is strongly genetically controlled, and a region of the genome located on chromosome XIX appears to contain a gene (genes) that controls gender determination, though there are noted examples of gender reversion and hermaphroditic plants in most species (Rottenberg et al. 2000; Markussen et al. 2007; Yin et al. 2008; Gaudet et al. 2008; Pakull et al. 2009, 2011; Paolucci et al. 2010). The peritelomeric region on chromosome XIX in female *Populus trichocarpa* genotypes contains approximately 1 Mb of DNA that is not found in male genotypes and appears to have a region of suppressed and/or reduced recombination that extends 3–4 Mb beyond the hemizygous segment in females (Yin et al. 2004a).

These observations suggest that in *P. trichocarpa*, gender is determined using a ZW system where the female genotype is the heterogametic gender (Yin et al. 2008). However, work by Pakull et al. (2011) suggested that both ZZ/ZW (female heterogamety) and XX/XY (male heterogamety) gender-determining systems could be present in some members of the genus *Populus*. Gender-determining systems in plants, in

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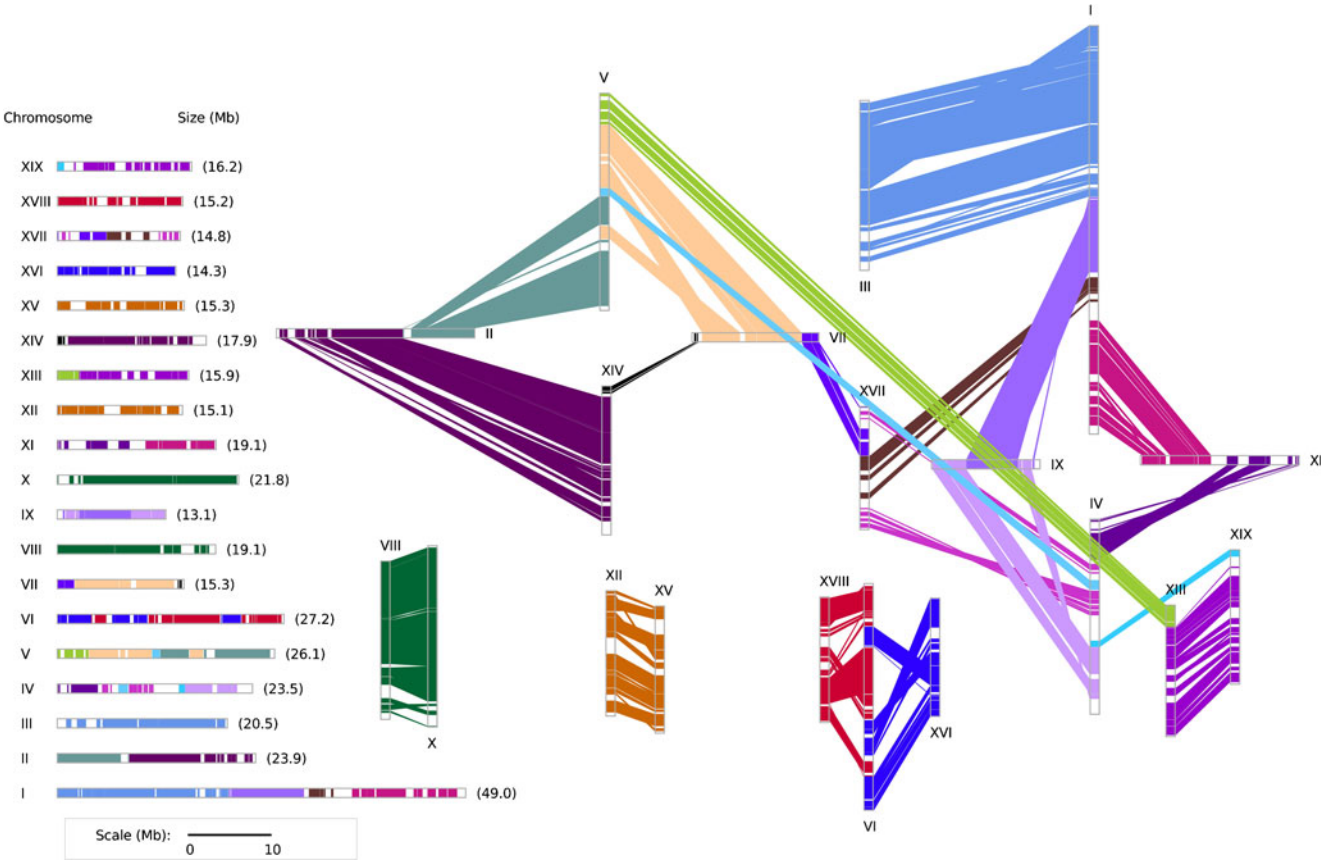

Fig. 1 The "salicoid" duplication event revealed by the *Populus* genome assembly version 2.2. *Common colors* refer to homologous genomic blocks. Chromosomes are identified by their linkage group number (*I* to *XIX*). The diagram to the left uses the same color-coding and further illustrates the chimeric nature and origin of most chromosomes. Chromosome XIX contains high homology with chromosome XIII expected for the peritelomeric end of XIX which contains the segment related to gender determination

general, exhibit a wide range of diversity to the extreme where some taxa have evolved systems that are unique to those taxa, e.g., in dioecious *Rumex* species, two different sex chromosomal systems and sex-determining systems have been described as: (1) XX/XY with an active Y chromosome (e.g., *Rumex acetosella*) and (2) XX/XY1Y2 with sex

Table 1 Sex ratios in *Populus* species and hybrids

References	Species/hybrid[a]	No. of trees		Sex		Segregation	
		Total	Flowering (%)	No. of males (%)	No. of females (%)	M/F ratio	χ^2 Ratio
Yin et al. (2008)	*Populus x canadensis*	312	312 (100)	197 (63.1)	115 (36.9)	2:1	21.5****
Paolucci et al. (2010)	*P. alba*	154	136 (88.3)	87 (64)	49 (36)	2:1	10.6**
Pakull et al. (2011)	*Populus x wettsteinii*	130	126 (96.9)	79 (62.7)	47 (37.3)	2:1	8.1**
Sabatti et al., unpublished data	*P. alba*	251	72 (28.7)	48 (66.7)	24 (32.3)	2:1	8.0**
Vanden Broeck, personal communication	*Populus x generosa*	140	70 (50)	49 (70)	21 (33.9)	2:1	11.2***
Vanden Broeck, personal communication	*P. x generosa*	137	62 (45.2)	41 (66.1)	21 (33.9)	2:1	6.45*
Gaudet et al. (2008)	*P. nigra*	165	118 (71.5)	63 (53.4)	55 (46.6)	1:1	0.5*****
Vanden Broeck, personal communication	*P. x canadensis*	141	53 (37.6)	21 (39.6)	32 (60.4)	1:1	2.3*****

Numbers of flowering trees with male and female flowers are presented

*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$; values are significant at their respective levels; *****$p > 0.5$; values are nonsignificant at this level

[a] *Populus x canadensis*, *P. deltoides* × *P. nigra*; *P. x generosa*, *P. deltoides* × *P. trichocarpa*; *P. x wettsteinii*, *P. tremula* × *P. tremuloides*

determination based on the X/A (autosome) ratio (e.g., *Rumex acetosa*) (Navajas-Pérez et al. 2005). There is also a species, *Rumex hastatulus*, which has two chromosomal races: the Texas race with XX/XY and the North Carolina race with XX/XY1Y2. In this species, the X/A ratio regulates sex determination, although the presence of the Y chromosome is necessary for male fertility (Smith 1963).

The chromosomes of *Populus* are typically metacentric and small (Blackburn and Harrison 1924; Meurman 1925; Erlanson and Hermann 1927; Nakajima 1937; Islam-Faridi et al. 2009), and based on cytological studies, there is no evidence of morphologically differentiated sex chromosomes in any *Populus* species (Peto 1938; Van Dillewijn 1940; van Buijtenen and Einspahr 1959). One generalized hypothesis is that sex chromosomes originate from autosomes, and dioecy almost certainly evolves from ancestral hermaphrodites that lacked sex chromosomes (Muller 1914; Liu et al. 2004). Based on genetic mapping results, evidence of sex chromosomes has been reported in various species by Gaudet et al. (2008), Yin et al. (2008), Pakull et al. (2009, 2011), and Paolucci et al. (2010). Yin et al. (2008) described genetic and genomic features in the peritelomeric region of chromosome XIX that suggested this region of the *Populus* genome is in the process of developing characteristics of a sex chromosome. A provocative feature of chromosome XIX is the location of a gender determination locus, which maps to alternate positions on chromosome XIX, depending upon the *Populus* species (Gaudet et al. 2008; Pakull et al. 2009, 2011; Yin et al. 2008; Paolucci et al. 2010) (Fig. 2), with a peritelomeric localization in members of the *Aigeiros* and *Tacamahaca* subgenera and a centromeric localization in *Leuce*.

Although mapping studies in *Populus* revealed that there is a single locus that is associated with gender determination,

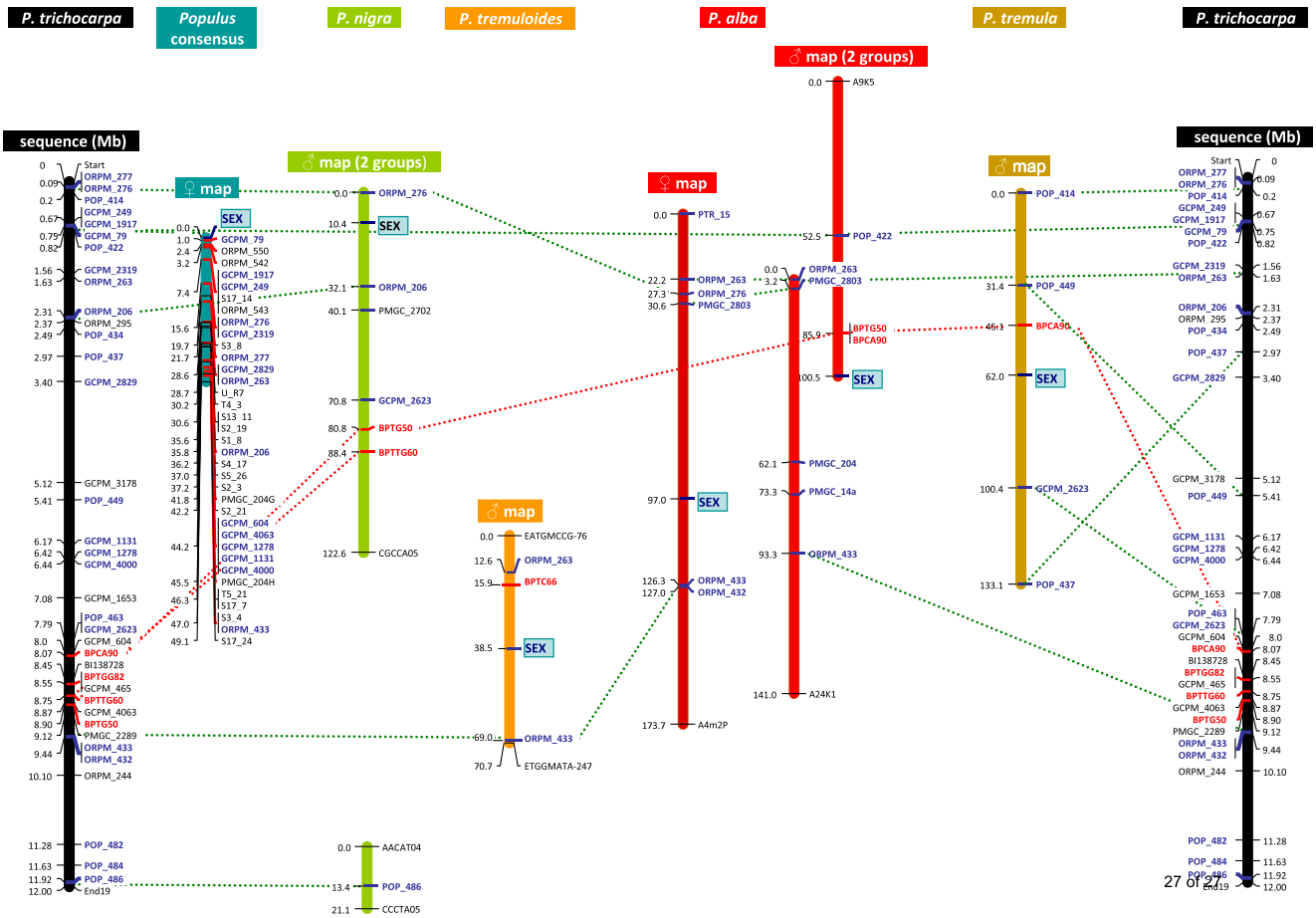

Fig. 2 Comparative mapping of chromosome XIX for gender locus position from different *Populus* species. Marker positions and distances on *P. trichocarpa* genome sequence v2.2 are proportional and drawn in *black*. Lengths of chromosome linkage groups are proportional to map distance in cM. *Numbers on the left of each chromosome* indicate the marker position. The consensus map (*P. trichocarpa* and *Populus deltoids*; Yin et al. 2008) is drawn in *blue*; the *P. nigra* map (in *green*), the *Populus tremuloides* map (in *orange*), the *Populus alba* map (in *red*) (Gaudet et al., unpublished), and the *Populus tremula* map (in *mustard*) (Pakull et al. 2009, 2011). Common SSRs are connected with *broken line*. SSR in *blue* were mapped in almost one parent of the pedigrees analyzed. The *red* SSRs were found linked to gender by Pakull et al. (2011)

recombination suppression, as noted above, would render all genes within this region as one locus. Thus, the gender locus might encompass several genes underlying gender determination. In support of this supposition, *Populus* trees show evidence of labile sex expression (Stettler 1971; Rowland et al. 2002). If there is more than one genetic locus determining gender, recombination would impair sexual differentiation. When the recombination suppression is relaxed or translocation of sex determination loci occurs, hermaphrodites may arise (Ming et al. 2007). The observed sex ratios in certain genetic backgrounds of *Salix viminalis* suggest a multilocus epistatic model of gender determination (Alström-Rapaport et al. 1998). To maintain separate sexes, the genes determining maleness or femaleness would have to be closely linked on opposing haplotypes of a single chromosome, and this region would have to develop local mechanisms to prevent recombination (Ohno 1967; Nei 1969; Charlesworth 1984; Ming et al. 2007).

Multiple lines of evidence from studies focused on *P. trichocarpa* support the role of chromosome XIX in sex determination. First, the sequenced tree, Nisqually-1, is a female, and it showed highly divergent haplotypes in the sex determination region (Tuskan et al. 2006). Second, suppressed recombination in this region was only observed in the female parent. Finally, distorted segregation ratios have been observed in the maternal genotypes of several mapping populations in *Populus* (Yin et al. 2004a). However, the gender-determining locus has not been resolved into an individual gene (genes) yet. As noted above, in *P. trichocarpa* and other members of the *Aigeiros* and *Tacamahaca* subgenera, the gender locus is located in peritelomeric region of chromosome XIX. However, in members of the *Leuce* subgenera, the gender-determining locus appears to be located near the centromere (Fig. 2). A segmental inversion on the maternal haplotype of chromosome XIX or a completely unique set of genetic loci in the centromeric region of the *Leuce* species may account for this difference and may be one of the reasons that members of the *Leuce* subgenera are generally not sexually compatible with other subgenera in interspecific crosses (Liesebach et al. 2011). Interestingly, in contrast with *P. trichocarpa*, a member of the *Tacamahaca* subgenera, an XY sex determination system appears to be in place in the *Aigeiros* and *Leuce* species.

The *Populus* genome was sequenced, assembled, annotated, and released in 2006, and at the time, represented the most polymorphic genome to be assembled using whole-genome shotgun approaches (Tuskan et al. 2006). A second draft assembly and annotated gene set was released in 2010 (http://www.phytozome.net/poplar), and the assembled sequence now captures roughly 83 % of the nucleotide (nt) space, and approximately 43,000 predicted gene models have been used to create and inform whole-genome microarray studies (Jansson and Douglas 2007), saturated genetic maps/QTL studies (Yin et al. 2008), and proteomics reference libraries (Abraham et al. 2011).

From a genome resequencing effort, we discovered that the gender-linked peritelomeric region of the chromosome XIX contains significantly fewer single nucleotide polymorphisms (SNP) than the rest of *Populus* genome (Fig. 3). There are approximately 1.8 SNPs per 1 kb of sequence at the peritelomeric end of chromosome XIX versus 2.6 SNP per 1 kb on the average across the entire genome. The peritelomeric end of chromosome XIX also appears to have a distinct evolutionary history compared to the rest of the genome as demonstrated by its alignment to the *Vitis* genome (Jaillon et al. 2007, Fig. 2a), where the peritelomeric end of chromosome XIX distinctively lacks homology with genomic segments found in the *Vitis* genome. The peritelomeric end of chromosome XIX also lacks homology with any of the duplicated segments associated with salicoid duplication (Fig. 1).

In addition, the peritelomeric end of chromosome XIX contains the largest cluster of the nucleotide-binding site–leucine-rich repeat (NBS–LRR) class of disease resistance genes in the entire *Populus* genome (Tuskan et al. 2006; Kohler et al. 2008). NBS–LRR genes function in the detection of pathogen occurrence and convey disease resistance signaling to activate gene expression. There is also a disproportionately high occurrence of small (18–24 nt) microRNAs (miRNA) on chromosome XIX coincident to the region containing the putative gender-determining locus and the major cluster of NBS–LRR genes (Klevebring et al. 2009). Such miRNAs are a class of posttranscriptional negative regulators that play a vital role in plant development and growth (Jones-Rhoades et al. 2006; Shuklaa et al. 2008; Henderson et al. 2006; Chan et al. 2005). A number of the identified miRNA are predicted to target the NBS–LRR disease resistance genes within the peritelomeric region of chromosome XIX. Furthermore, the NBS–LRR genes have been significantly expanded in *Populus* relative to *Arabidopsis* (Kohler et al. 2008; Meyers et al. 2003).

The hypothesis

Based on the above information, the following is a working hypothesis for the evolution of an incipient sex chromosome in *Populus*. Specifically, prior to the advent of the crown taxa of the family Salicaceae, there was a common progenitor of all modern Salicaceae species that was monecious, diploid, capable of vegetative reproduction via adventitious root formation, and susceptible to pathogenic attack isolated to its stigma and/or style. The pathogen caused complete necrosis of all female reproductive structures such that sexual reproduction was disrupted, favoring genes that facilitated the establishment and promulgation of vegetative

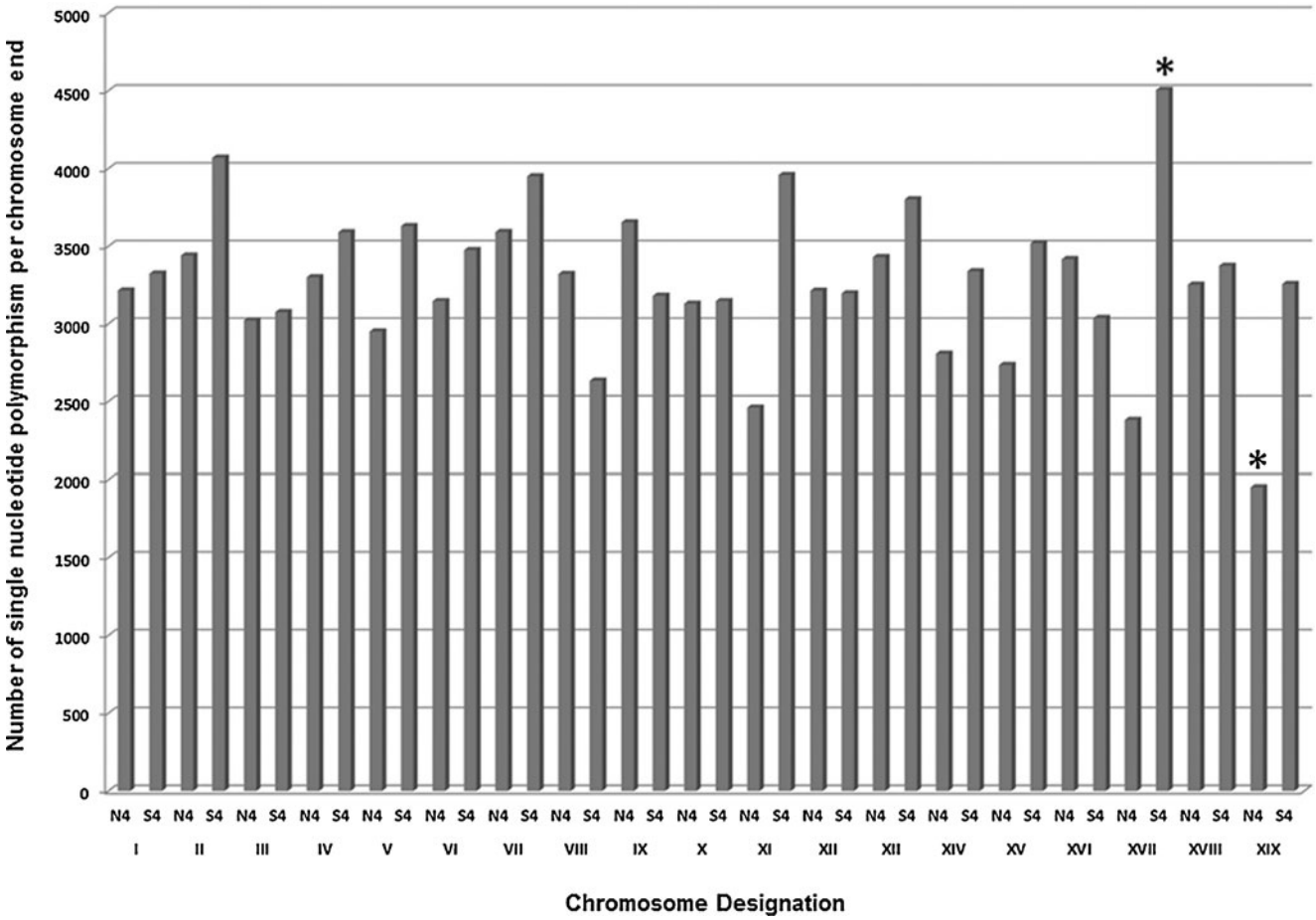

Fig. 3 Single nucleotide distribution across all 19 *Populus* chromosomes, with *N* and *S* representing an arbitrary 4 Mb "north" and "south" end of each chromosome. The S4 and N4 regions of chromosomes XVII and XIX contain significantly ($p \leq 0.01$) more and fewer SNP than expected by chance alone, respectively

propagation. Extended interannual vegetative propagation allowed time for somatic mutations to occur, experience selection pressure, and accumulate favorable alleles in the susceptible progenitor genotype. Favorable mutations at multiple independent loci then formed the basis of resistance to the putative female-specific floral pathogen. Selective pressure from the putative pathogen also facilitated the accumulation of miRNA targets within transcripts of the genes responsible for this resistance as a means of regulating gene expression. Over evolutionary time, small translocations within the progenitor genome disproportionately resulted in the accumulation of resistance genes on the peritelomeric portion of chromosome XIX, which then minimized linkage disequilibrium between the individual resistance loci required for female organ function and survival. Suppressed recombination associated with the telomere reduced the genetic load on the progeny and allowed these genes to be inherited as a haplotypic block. Translocation of genes related to female organogenesis to the peritelomeric end of chromosome XIX ultimately resulted in dioecy. All modern taxa in the family Salicaceae, descendants of this progenitor, are now dioecious, capable of vegetative propagation, and have syntenic chromosome structure. Selective sweep associated with these events is evident in the reduced number of single nucleotide polymorphisms in the peritelomeric region of chromosome XIX.

Evidence in the modern *Populus* genome

The lack of comparative synteny on chromosome XIX with *Vitis*, the suppressed recombination in the peritelomeric end of chromosome XIX, and the reduced number of SNP found in this region are consistent with the occurrence of a selective sweep in *Populus*. Reduction in the accumulation of genetic mutations is a classic indication of an ancient severe genetic bottleneck within the evolution history of an organism or segment of DNA. Paape et al. (2008) reported a similar event in Solanaceae where the founding members of the extant genera *Physalis* and *Witheringia* appear to have been derived from a reduced number of lineages in genomic regions surrounding the S-locus for self-incompatibility. The

fingerprints of restricted founding lineages and genetic bottleneck are also present in many modern domesticated crop plants where artificial selection has reduced the amount of genetic variation surrounding loci associated with domestication (Purugganan and Fuller 2009; Hyten et al. 2006; Palaisa et al. 2004). Based on nucleotide diversity, the peritelomeric end of chromosome XIX appears to be younger than the rest of the *Populus* genome. Interestingly, similar observations have been made for the X chromosomes in humans and mice (Hughes et al. 2010; Patterson et al. 2006).

Floral pathogens and floral biochemistry

Many species of plants have evolved methods of producing antibacterial and antifungal compounds in their floral tissues (Tavares et al. 2008; Jones and Dangl 2006; Lokvam and Braddock 1999; Carlson et al. 1948). These compounds are thought to protect the flowers from lethal and semilethal attacks from microorganisms (Theis et al. 2009; Thadeo et al. 2008; Vamosi and Otto 2002). Moreover, many of these compounds vary between tissue types, flower structures, gynomorphs, and andromorphs (Kaltz and Shykoff 2001; Strauss 1997; Carlson et al. 1948). In addition, there are several plant diseases that are known to affect only the male or female structures of perfect flowers or male or female flowers in dioecious plant species (Giles et al. 2006; Lokvam and Braddock 1999). Finally, there are plant pathogens that gain access via the stigma or style tissue within female flowers (Pusey and Curry 2004; Stretch and Ehlenfeldt 2000; Shykoff et al. 1997).

Populus and other members of the Salicaceae family are known to produce antimicrobial and antifungal compounds in nectary found near meristematic tissues, and that these compounds contribute to plant adaptive success (Heil 2008; Thadeo et al. 2008). A gas chromatography-mass spectrometry (GC-MS) analysis of the metabolomic profiles of expanding floral buds of 16 trees (12 females and 4 males) from two *Populus* species (*Populus deltoides* and *Populus nigra*) was conducted to determine whether secondary metabolites were correlated with gender (Table 2). Species differences in secondary metabolites of *Populus* are well documented (Greenaway and Whatley 1990, 1991a, b; Greenaway et al. 1989, 1992; Tsai et al. 2006), and male and female flowers of *Salix* display gender-specific floral scent characteristics (Fussel et al. 2007). Our analyses demonstrate metabolite differences between genders, that is, although buds of both species and both genders contain pinobanskin-3-acetate, pinobanksin-3-acetate chalcone and pinobanksin and 3-isobutanoate, the male floral buds contained very low concentrations of these metabolites. In contrast, *P. nigra* males contained high concentrations of higher-order populin conjugates, including 6-hydrocyclohexenyl (HCH)-populin and populin conjugated with benzoic acid and another unknown 476 Da moiety. Moreover, several secondary metabolites were orthogonally associated with gender. This included major secondary metabolites of *Populus*, including salicortin and salireposide, which were higher in females. There were also several unidentified secondary metabolites that were much higher in female buds, including a metabolite tentatively identified as caffeoyl-populoside and a series of metabolites that all share a 171 m/z moiety, along with a coumarate glycoside conjugate (retention time (RT) 18.78 min) and a caffeoyl glycoside conjugate (RT 19.3 min). Scanning for the unidentified 171 m/z moiety resulted in the identification of several additional secondary metabolites uniquely associated with female floral buds, including a feruloyl glycoside conjugate (RT 19.36 min) and a benzyl-caffeoyl glycoside conjugate (RT 20.3 min). Taken together, these preliminary analyses suggest that there are gender-specific accumulations of phenolic glycosides. Based on these results, antimicrobial and antifungal compounds appear to be differentially found in male and female floral structures.

LRR genes

As a perennial organism, *Populus* species interact with numerous diverse microorganisms over periods of decades (Gottel et al. 2011). In order to survive, *Populus* species must resist against recurring, perpetual pathogenic attack. Moreover, with a large root system maintaining water and

Table 2 Concentrations (ug/g FW sorbitol equivalents) of *Populus* floral bud metabolites associated with gender

Species	Gender	Salicortin	Salireposide	18.78–171 331 Coumaroyl glycoside	19.30–171 331 Caffeoyl glycoside	19.36–171 Feruloyl glycoside	20.30–171 Benzyl-caffeoyl glycoside	22.06 Caffeoyl-populoside
P. nigra	Female	2,110.5 (210.8)	116.3 (16.1)	43.1 (4.7)	11.8 (3.3)	41.8 (4.4)	17.1 (3.5)	28.3 (11.7)
P. deltoides	Female	1,991.7 (145.6)	100.9 (13.9)	31.6 (4.2)	13.8 (1.6)	37.5 (6.0)	10.7 (3.8)	32.3 (6.7)
P. nigra	Male	488.3 (143.2)	13.9 (3.8)	1.9 (0.3)	0.3 (0.2)	0 (0)	0 (0)	0.2 (0.2)
P. deltoides	Male	1,243.0 (97.4)	45.7 (4.5)	9.2 (8.2)	5.1 (0.6)	20.6 (0.7)	0 (0)	9.7 (3.1)

As determined by gas chromatography-mass spectrometry; retention time and key m/z are shown at the column headings; mean and SEM () of two male to six female replicate buds are shown within rows

nutrient uptake and functioning to facilitate interannual store of carbon over multiple decades, the selective pressure to develop symbiotic interactions with soil microflora may be as strong as to develop pathogenic resistances. As an illustration, various loci controlling variation for both symbiosis levels and resistance have been identified in *Populus* (Zhang et al. 2001; Yin et al. 2004b; Jorge et al. 2005; Tagu et al. 2005; Kohler et al. 2008; Duplessis et al. 2009; Labbé et al. 2011).

Plants, in general, and *Populus*, in particular, have a variety of disease resistance genes (R) encoding proteins involved in the detection of pathogens and herbivores. The largest class of R–genes encodes intracellular nucleotide-binding site–leucine-rich repeat proteins, and they are divided into two main subfamilies (TNL and TIR) based on their predicted N-terminal protein domains (McDowell and Wolffenden 2003). NBS–LRR genes are known to trigger protease inhibitor activity; control protease, chitinase, and kinase production; and regulate salicylic acid, jasmonic acid, ethylene, and nitric oxide signaling (Duplessis et al. 2009; Sánchez-Rodríguez et al. 2009; McHale et al. 2006; Meyers et al. 1999, 2003; McDowell and Wolffenden 2003). In *Populus*, 37 NBS–LRR genes are found in the peritelomeric region of chromosomes XIX and represents nearly 10 % of all NBS–LRR found throughout the rest of the genome (Tuskan et al. 2006; Kohler et al. 2008, Fig. 1).

Genome-wide, roughly 400 NBS–LRR genes have been identified in *Populus*, which is approximately double the number identified in *Arabidopsis* (Kohler et al. 2008). The higher number in *Populus* appears to represent an expansion in *Populus* rather than a contraction in *Arabidopsis* (Meyers et al. 2003; Tuskan et al. 2006; Kohler et al. 2008). Indeed, a remarkable feature of plant NBS–LRR genes is their genomic organization in multigene clusters (Kohler et al. 2008; Yang et al. 2008). In *Populus*, these clusters are distributed unevenly over the chromosomes and in clusters of clusters or "superclusters" of which three occur on chromosome XIX. Highly similar sequences in head-to-tail orientation suggest that intralocus recombination gave rise to the translocation of a sequence block (Richy et al. 2002). The largest NBS–LRR gene supercluster on *Populus* chromosome XIX collocates with the resistance loci *MER*, *R1*, and *RUS*, conferring qualitative or quantitative resistance to *Melampsora larici-populina* (Lescot et al. 2004; Jorge et al. 2005; Bresson et al. 2011). Thus, ancient segmental duplication and subsequent chromosomal rearrangement that accounted for 10 % of the amplification of NBS superclusters suggests positive selective pressure on chromosome XIX that may expedite the progression of sex chromosome evolution.

Short RNAs

In *Populus*, there are still only a small number of publications that have examined or profiled miRNAs, and only one that has examined the entire sRNA population (Klevebring et al. 2009). An intriguing finding from the data presented in Klevebring et al. (2009) was that the proposed sex-determining peritelomeric region of chromosome XIX showed a distinctive pattern of sRNA occurrence that differed significantly from the rest of the genome. Within this region, there was distinct overrepresentation of 21 and 24 nt sRNAs along with a phased siRNA locus. Target prediction of the phased siRNAs indicates that they target NBS–LRR genes within the same region of chromosome XIX.

Using the methodology outlined in Klevebring et al. (2009), but applied to v2.2 of the *P. trichocarpa* genome assembly (http://www.phytozome.net/poplar), we recharacterized sRNA occurrence. Based on our reanalysis, there was an above average occurrence of sRNAs from the upper 1 Mb of chromosome XIX, in particular, 21 and 24 nt sRNAs (as was reported in Klevebring et al. 2009). Because the reference genome represents a female *P. trichocarpa* genotype, it is not known whether this pattern is present in both males and females, or whether there are sex-specific differences in the sRNA population produced from this region. Such patterns have not been reported in other species.

In contrast to the v1.1 analysis, five phased loci were identified on chromosome XIX with four of the five located within the peritelomeric end of chromosome XIX (e.g., Fig. 4). Target prediction of these four loci identified near-exclusive targeting of NBS–LRR genes with almost all target genes located within the peritelomeric region of chromosome XIX. Deeper sequencing and profiling of additional tissues and developmental states will be needed to clarify these results, as read counts for nearly all sequences within the loci were low (<5); however, it appears that alternate male and female haplotypes may have haplotype-specific phased loci that target NBS–LRR genes in a haplotype-specific manner.

Many miRNA families regulate the development in *Arabidopsis* and have been shown to be necessary for proper specification of floral organ identity (e.g., miR172; Jones-Rhoades et al. 2006; Mallory and Vaucheret 2006). *Arabidopsis* plants that over express miR172 have floral defects that resemble APETALA2-like loss-of-function mutants where there is an absence of petals and sepals and an excess of carpels (Aukerman and Sakai 2003; Chen 2004). Moreover, recent studies investigating genes that control sex determination in maize reveals that a miRNA is involved in the determination of the male inflorescence (Banks 2008). Here, the tasselseed4 miRNA, i.e., miR172, targets APETALA2 floral homeotic transcription factors (Chuck et al. 2007). miR172 also targets F-box family protein (FKF1) in *Arabidopsis*, and in *Eschscholzia californica*, miR172 appears to control protein degradation and sex determination (Barakat et al. 2007b). Interestingly, the miR172 family

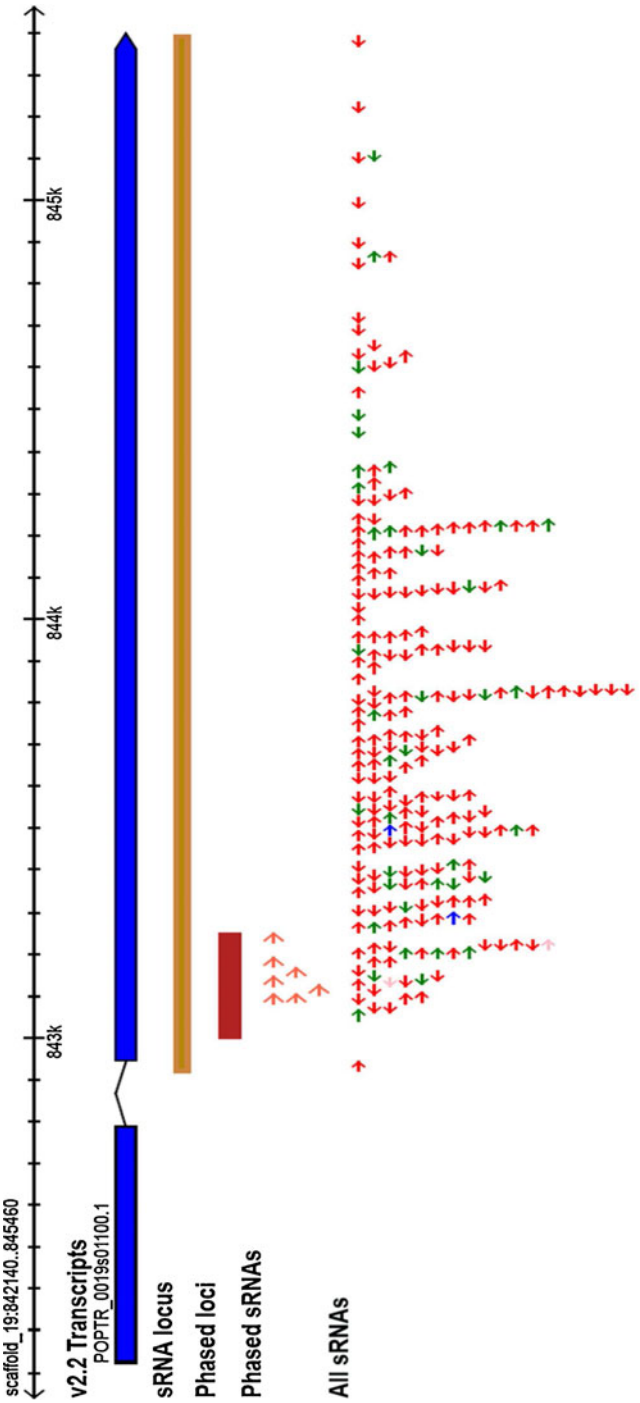

Fig. 4 A phased sRNA locus located on chromosome XIX. The phased locus is located within the second exon of POPTR_0019s01100, a gene containing multiple leucine-rich repeat domains. The sRNA locus and phased locus shown were identified using the UEA plant sRNA toolkit (http://srna-tools.cmp.uea.ac.uk/). Phased sRNA sequences are shown as are all sRNA sequences within the identified sRNA locus. sRNA *sequence colors* indicate size class: *pink* ≤19 bp, *red* ≤21 bp, *green* ≤23 bp, *blue* ≤25 bp. All sRNA data are from Klevebring et al. (2009)

is conserved in *Populus* (Barakat et al. 2007a) and is located on the peritelomeric end chromosome XIX, and coincidentally, there is an under representation of F-box genes in the peritelomeric region of chromosome XIX in *Populus* (Yang et al. 2008, Fig. 1).

Future characterizations of the role of miRNAs in sex determination in *Populus* may illuminate biochemical preferences in the sex determination pathway and, in the process, define male/female differentiation. Deep sequencing of miRNAs from pre- and postdifferentiated male and female floral meristems will be necessary to understand which miRNAs change during differentiation and to determine the target gene upon which they act.

The metabolic differences between male and female flowers, the overrepresentation of NBS–LRR genes, the presence of phased sRNA loci targeting those genes, and the generally higher than average production of sRNAs, all which collocate with gender, support the hypothesis that resistance to and regulation of a floral pathogen and gender determination coevolved in a region of the genome that experiences reduced recombination, i.e., the peritelomeric region of chromosome XIX in *P. trichocarpa*.

Conclusions

Genetic determination of gender occurs in most plant species and is a fundamental developmental and evolutionary process. The sexual phenotypes of commercially important *Populus* species and their varieties will dictate how they are bred and cultivated. Understanding of the genetic mechanisms guiding this intricate process is in its infancy. Thus, dissecting the mechanisms underlying gender determination in *Populus* will allow several evolutionary, developmental, and economic questions to be resolved. Based on various *Populus* species, we suggest that the gender determination may vary among species, with some species following a ZW gender determination system and other using a XY system. Identifying the molecular basis of floral differentiation that cosegregate with gender represents a promising approach to define the gender-determining system in *Populus*. Resequencing and expression studies in parental, F_1, and F_2 generations will enable us to definitively identify the heterogametic sex.

While genetic linkage mapping studies have started to reveal regions of recombination suppression in *Populus*, identifying the actual gene or genes involved in gender determination remains a prime objective. Greater effort in comparative mapping, targeted resequencing, gender-specific expression studies, and physical mapping efforts will be invaluable in discovering the key gene or genes and in answering questions about their evolution. These types of information will shed light on the developmental patterns of gender determination and evolution of sex chromosomes. Finally, cloning the *Populus* gender-determining genes and complementation transgenic experiments will

ultimately be needed to unravel the role of genetic and epigenetic factors in gender determination. The genus *Populus* is an excellent model for studying the evolution of gender determination because of the genus-wide occurrence of dioecy-related gender-determining systems that can provide new perspectives on the genetic mechanism of gender determination in plants in general. With the availability of the whole-genome sequence and the initiation of a number of efforts to characterize adaptive polymorphisms and gender determination in natural and structured populations, answers to these and other intriguing questions about the evolutionary biology of this model genus will emerge in the near future.

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